

MINIREVIEWS

RNA Interference in Fungi: Pathways, Functions, and Applications[▽]

Yunkun Dang, Qiuying Yang, Zhihong Xue, and Yi Liu*

Department of Physiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390

Small RNA molecules of about 20 to 30 nucleotides function in gene regulation and genomic defense via conserved eukaryotic RNA interference (RNAi)-related pathways. The RNAi machinery consists of three core components: Dicer, Argonaute, and RNA-dependent RNA polymerase. In fungi, the RNAi-related pathways have three major functions: genomic defense, heterochromatin formation, and gene regulation. Studies of *Schizosaccharomyces pombe* and *Neurospora*, and other fungi have uncovered surprisingly diverse small RNA biogenesis pathways, suggesting that fungi utilize RNAi-related pathways in various cellular processes to adapt to different environmental conditions. These studies also provided important insights into how RNAi functions in eukaryotic systems in general. In this review, we will discuss our current understanding of the fungal RNAi-related pathways and their functions, with a focus on filamentous fungi. We will also discuss how RNAi can be used as a tool in fungal research.

RNA interference (RNAi) is a conserved eukaryotic gene-silencing mechanism, and its discovery is one of the most important scientific breakthroughs in the past 20 years. In various RNAi pathways, small noncoding RNAs (sRNAs) of about 20 to 30 nucleotides (nt) act as regulators of cellular processes, such as development, RNA stability and processing, host defense, chromosome segregation, transcription, and translation (11, 12, 27, 34, 57). The silencing effects mediated by sRNAs in different pathways require an Argonaute/Piwi protein as the core component of the RNA-induced silencing complex (RISC) (11, 16). Upon loading of the sRNAs, RISC is guided to the target RNAs, and silencing is triggered.

Eukaryotic sRNAs are generally categorized into three main classes: short interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs). In terms of biogenesis, siRNAs and miRNAs are both derived from double-stranded RNA (dsRNA) precursors that are recognized and processed by Dicer to generate short duplexes (21 to 25 nt). The siRNAs and miRNAs are different in two key aspects. First, siRNAs are derived from exogenous dsRNA (e.g., viral RNA) or endogenous transcripts from repetitive sequences (e.g., transposable elements), or from transcripts that can form long hairpins (12, 34, 41). In many organisms, siRNA-induced silencing requires RNA-dependent RNA polymerases (RdRPs) to generate dsRNA from single-stranded RNA (ssRNA) or to amplify sRNA signals (2, 24, 33). miRNAs, on the other hand, are generated from miRNA-encoding genes that generate ssRNA precursor transcripts that form hairpin structures. Second, siRNAs normally fully match their mRNA targets. The siRNAs trigger RNA cleavage or transcriptional silencing mediated by the Argonaute proteins and generally function in genome

defense. In contrast, miRNAs can target mRNAs that are not fully complementary and cause mRNA degradation and translational repression (3, 6).

piRNAs acquired their name due to association of these sRNAs with proteins from the Piwi clade of animal Argonaute proteins; these sRNAs have not been identified in organisms outside the animal kingdom. piRNAs are mostly derived from repetitive elements, transposons, and large piRNA clusters in germ cells (85). They have a distinct biogenesis pathway. piRNAs appear to be processed from single-stranded precursors and can be amplified through a “ping-pong” mechanism mediated by different Piwi family proteins (8, 38, 56, 61). piRNAs play important roles in maintaining germ cell genome stability, although the functions of many piRNAs are still unknown (12, 34, 48, 62, 84, 89).

RNAi components (Dicer, Argonaute, and RdRP) have been identified in all major branches of eukaryotes (16, 79), suggesting that RNAi is an ancient defense and/or regulatory mechanism that existed in the common eukaryotic ancestor. In addition, homologs of the Argonaute proteins, but not other core RNAi components, are found in some eubacteria and archaea (86). During evolution, the RNAi pathway has undergone adaptation, leading to novel biogenesis mechanisms and functions in different eukaryotic organisms and lineages. RNAi components are absent from some eukaryotic organisms, most notably *Saccharomyces cerevisiae*, indicating that the RNAi pathway is dispensable for some single-celled organisms.

The fungi kingdom is a major branch of the eukaryotic organisms and is estimated to include more than 1 million species, with enormous diversity in ecology, morphology, and life cycles. Studies of the filamentous fungus *Neurospora crassa* and the fission yeast *Schizosaccharomyces pombe* have uncovered key RNAi components and functions and contributed significantly to our understanding of RNAi in eukaryotes. In this review, we will discuss the RNAi pathways in fungi, mechanistic and functional adaptations in different organisms and environments, and how RNAi can be harnessed for basic research and industrial uses. Since the RNAi studies of *S. pombe*

* Corresponding author. Mailing address: Department of Physiology, ND13.214A, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9040. Phone: (214) 645-6033. Fax: (214) 645-6049. E-mail: Yi.Liu@UTSouthwestern.edu.

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have been reviewed previously (9, 36, 62, 90), this review will mostly focus on RNAi in filamentous fungi. It is worth noting that in the fission yeast, RNAi mediates gene silencing at the transcriptional level by heterochromatin formation, whereas in filamentous fungi RNAi mostly results in posttranscriptional gene silencing and has no confirmed role in transcriptional gene silencing (19, 30).

RNAi IN FUNGI

The fungi kingdom consists of a large and diverse group of eukaryotes. Current research recognizes seven phyla and one subkingdom that consists of two phyla, *Ascomycota* and *Basidiomycota*. Based on current fungal taxonomy, yeasts and filamentous fungi do not form a monophyletic group (44). Most yeasts fall into the phyla *Ascomycota* and *Basidiomycota*, whereas most filamentous fungi belong to *Ascomycota* (37). Since the three core RNAi components (RdRP, Dicer, and Argonaute) have been found in species of different phyla, it is believed that the fungal common ancestor possessed a functional RNAi pathway. In this pathway, an aberrant RNA would be first recognized and converted into dsRNA by RdRPs, subsequently processed by Dicers into siRNA, and loaded onto Argonaute proteins (64). However, this ancestral pathway has experienced various adaptations during fungal evolution. For example, in budding yeasts, the RdRP appears to be universally lost (28, 64): budding yeasts either have noncanonical RNAi or have no RNAi pathway at all. On the other hand, RNAi has largely been retained in all filamentous fungi examined, but it has diverse functions. In the model organism *Neurospora crassa*, siRNAs and microRNA-like small RNAs (miRNAs) function in genome defense and gene regulation (31, 57). RNAi in *Neurospora* functions in both vegetative and sexual stages, establishing it as a good paradigm for the diversity of fungal RNAi pathways and functions.

GENOME DEFENSE SYSTEM IN *NEUROSPORA CRASSA*

RNAi is an ancient host defense mechanism against viruses and transposons (27). The filamentous fungus *N. crassa* possesses several mechanisms to defend against viral and transposon invasion. Hyphae, the vegetative tissue of filamentous fungi, are typically divided into cells by internal cross-walls called septa. As septa harbor pores large enough for ribosomes, mitochondria, and nuclei to flow through, invading viruses are also able to quickly move from one cell to another (42). To battle against this threat, fungi have evolved an efficient antiviral mechanism. Although no virus has been found in *N. crassa* laboratory strains so far, dsRNA, an intermediate of viral RNA replication of many viruses, can initiate potent RNAi-mediated gene silencing in these strains (15, 18). In addition, the expression of dsRNA in *N. crassa* triggers a robust dsRNA-activated transcriptional program that activates the expression of all major RNAi genes, including the Argonaute proteins QDE-2 and Dicer, and many homologs of known antiviral genes (20). The induction of these genes by dsRNA suggests that RNAi is part of the conserved ancient defense response to viral infection.

N. crassa has three mechanisms, two of which are RNAi related, to suppress transposon invasion during the vegetative

(asexual) and sexual stages of its life cycle through recognition of repetitive and highly homologous sequences. In the vegetative stage, which accounts for most of the *Neurospora* life cycle, the introduction of repetitive DNA sequences triggers posttranscriptional gene silencing of all homologous genes, an RNAi silencing phenomenon known as quelling (74). During the sexual cycle, two distinct silencing mechanisms function: repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD; also referred to simply as meiotic silencing) (78, 82). RIP occurs during the early stage of the sexual cycle, when the fused cytoplasm still harbors two haploid nuclei from opposite mating types. RIP causes mutations from C · G to A · T in the repetitive sequences (78). MSUD is an RNAi-related mechanism that occurs later than RIP and is activated when two haploid nuclei are fused into a diploid nucleus. Mechanistic studies of quelling and MSUD have provided important information on how RNAi functions in eukaryotes.

QUELLING, A GENOME DEFENSE MECHANISM OF *NEUROSPORA* IN THE ASEQUAL CYCLE

Quelling was one of the first RNAi-related phenomena described in eukaryotes, and its study resulted in the identification of some of the earliest known RNAi components (74). In 1992, when Macino and his colleague transformed *N. crassa* with multiple copies of the structural genes *albino-1* (*al-1*) and *albino-3* (*al-3*) required for carotenoid biosynthesis (responsible for the orange pigment of *N. crassa*), they obtained some pale yellow/white transformants that had decreased *al* mRNA levels (74). They named this posttranscriptional gene-silencing phenomenon quelling. In their subsequent studies with forward and reverse genetics approaches, several genes coding for components of the quelling machinery, including quelling deficient element genes *qde-1*, *qde-2*, and *qde-3* and Dicer protein genes *dcl-1* and *dcl-2*, were identified (13, 15, 23–25). *qde-1* encodes an RdRP and was the first RNAi component identified in a eukaryotic organism (24). The identification of QDE-1 soon after the landmark study of *Caenorhabditis elegans* by Fire, Mello, and colleagues (29) provided strong *in vivo* evidence that demonstrated the role of dsRNA in RNAi. *qde-2*, *dcl-1*, and *dcl-2* encode an Argonaute protein and two Dicer-like proteins, respectively (13, 15). These four proteins are the core components in the quelling pathway, and they are highly conserved in most eukaryotes. In short, the aberrant RNAs (aRNAs) produced from a repetitive transgene locus are specifically recognized by QDE-1, which synthesizes dsRNAs. These dsRNAs are then cleaved by DCL proteins into duplex siRNAs of ~25 nt and subsequently loaded onto QDE-2, the core component of RISC (14, 31). In vegetative cells, DCL-2 is the major Dicer enzyme that processes dsRNA into siRNA, but DCL-1 has a redundant role (15).

The siRNA duplex-bound Argonaute protein is initially inactive. To allow recognition and cleavage of its mRNA targets, the passenger strand of the siRNA duplex must be removed from the complex. In *N. crassa*, the activation of RISC is a two-step process, requiring the slicer activity of QDE-2 and the QDE-2-interacting protein QIP, an exonuclease (60). First, the QDE-2-bound double-stranded siRNA duplex is nicked by

QDE-2 using its slicer activity: QDE-2 treats the passenger strand of the siRNA as a substrate. Second, QIP, which is recruited by QDE-2, recognizes the nicked passenger strand of siRNA and degrades it with its exonuclease activity. QIP was the first identified exonuclease required for RNA-induced silencing in eukaryotes. The importance of cleavage activity of Argonaute proteins in siRNA duplex strand separation was later demonstrated for other organisms (10, 87), and it appears to be an essential step in RNAi pathways. Recently, the *Drosophila melanogaster* RNase C3PO was shown to remove the nicked siRNA passenger strand to activate the RNAi pathway (59).

Although the mechanism of quelling downstream of dsRNA production is largely clear, how cells generate repetitive transgene-specific dsRNA is still poorly understood. To trigger quelling, the ectopic sequences homologous to the endogenous counterpart must exceed the threshold length of about 130 nt (22). In addition, a transgene *per se* is not sufficient to trigger quelling unless the transgenic sequences are arranged in large tandem arrays. Based on these observations, it was hypothesized that the large tandem repeats may produce aberrant DNA secondary structures that are recognized by QDE-3, a *recQ* DNA helicase and an essential component of quelling (25). But how QDE-3 is involved in the production of aRNA and how QDE-1 specifically recognizes the aRNA to produce dsRNA are not known. Studies of the plant RNAi pathway suggest that misspliced or decapped mRNAs may be recognized as aRNAs and trigger RNAi (32, 43). However, the recent studies on DNA damage-induced qiRNA (discussed below) and quelling in *N. crassa* suggest the existence of a different mechanism. Surprisingly, the production of aRNAs after DNA damage does not require the typical DNA-dependent RNA polymerases (DdRPs), such as polymerase I (Pol I), Pol II, or Pol III (54). Instead, QDE-1, the RdRP, is required for aRNA production (53, 54). Biochemical studies showed that QDE-1 is both an RdRP and a DdRP, and in fact, the DdRP activity of QDE-1 is much stronger than its RdRP activity (53). Therefore, in dsRNA production, QDE-1 has dual roles: it first uses its DdRP activity to produce aRNA from single-stranded DNA (ssDNA) and then uses its RdRP activity to convert aRNA into dsRNA. This aRNA production mechanism explains why QDE-1 specifically recognizes aRNA and not other cellular RNAs.

The function of QDE-1 in aRNA synthesis requires its recruitment to ssDNA. *In vitro*, QDE-1 produces mostly RNA/DNA hybrids from ssDNA templates (53, 54), indicating the existence of additional factors in aRNA and dsRNA synthesis. Nolan et al. showed that QDE-1 interacts with replication protein A (RPA), an ssDNA binding protein complex important in DNA replication, repair, and recombination pathways (71), but the role of RPA in quelling is not known. Recently, we showed that qiRNA production and aRNA production are completely abolished in an *rpa-3* knockout strain (53). Importantly, RPA-3 is also required for quelling, linking the mechanism of qiRNA production to that of quelling. *In vitro* RNA polymerase assays showed that in the presence of RPA, QDE-1 almost exclusively produces dsRNA from ssDNA, due to the lack of RNA/DNA hybrids. These results suggest that RPA functions in the quelling and qiRNA pathways by recruit-

ing QDE-1 to ssDNA and by preventing the formation of RNA/DNA hybrids.

Taken together, these data support a working model for dsRNA production in the quelling pathway that is summarized in Fig. 1. First, the presence of tandem repetitive DNA sequences results in the formation of aberrant DNA structures that are recognized by QDE-3. QDE-3 unwinds dsDNA to produce ssDNA, which is then stabilized by RPAs. QDE-1, recruited by QDE-3 and RPA to the ssDNA locus, uses its DdRP activity to produce aRNA from ssDNA, and then it converts the aRNA into dsRNA via its RdRP activity.

MEIOTIC SILENCING OF UNPAIRED DNA IN THE SEXUAL CYCLE OF *N. CRASSA*

Meiotic silencing of unpaired DNA is an RNAi-related phenomenon that can silence unpaired DNA/chromosome during the sexual cycle in *N. crassa* (4, 46, 63, 81, 82). Meiotic silencing occurs in diploid ascus cells, which are formed by the fusion of two nuclei of opposite mating types (82). The diploid cells eventually divide into eight haploid ascospores after two rounds of meiosis and one round of mitosis. Specifically, meiotic silencing acts in the prophase of first meiosis, when there is a region of unpaired DNA between the two homologous parental chromosomes (i.e., DNA exists in one parental chromosome but not in its pairing partner or heterologous DNA on parental chromosomes) (72, 81, 82). Meiotic silencing triggers the silencing of all homologous sequences in the genome, and this effect can last throughout the rest of the sexual cycle. The unpaired DNA can be caused by the deletion or addition of DNA in one parental strain. Thus, meiotic silencing serves as a complementary mechanism to RIP, which inhibits transposon replication during the sexual cycle. It should be noted that both RIP and meiotic silencing can occur during the sexual stage if repetitive DNA also results in unpaired DNA on the homologous chromosome. In addition, the ability of meiotic silencing to detect and silence transcription from unpaired DNA may also provide a safeguard against genomic rearrangement events, such as deletions, duplications, and translocations, in the sexual cycle.

The fact that meiotic silencing can silence expression of other homologous genes in *trans* even if those copies are paired (4, 81, 82) suggests that the silencing signal is *trans*-acting, similar to the case in quelling. Indeed, genetic studies have identified a set of critical genes that are highly homologous to the components of the quelling pathway but are specifically expressed in the meiotic stage. *Sad-1* (suppressor of ascus dominance 1) is the first known meiotic silencing component identified by screening for mutants deficient in meiotic silencing. *SAD-1* is a putative RdRP homologous to QDE-1 (82). The identification of *SAD-1* indicated that, like quelling, the production of dsRNA is an essential step in meiotic silencing. *SMS-2* (suppressor of meiotic silencing 2), identified by reverse genetics, is an Argonaute protein homologous to QDE-2 (52). *SMS-2* is thought to function by binding to sRNAs originating from the unpaired DNA region to trigger posttranscriptional silencing. Although both DCLs have somewhat redundant functions in the quelling pathway, DCL-2 is the major Dicer enzyme active in the quelling pathway. For meiotic silencing, however, only DCL-1 (also called *SMS-3*) is required, suggest-

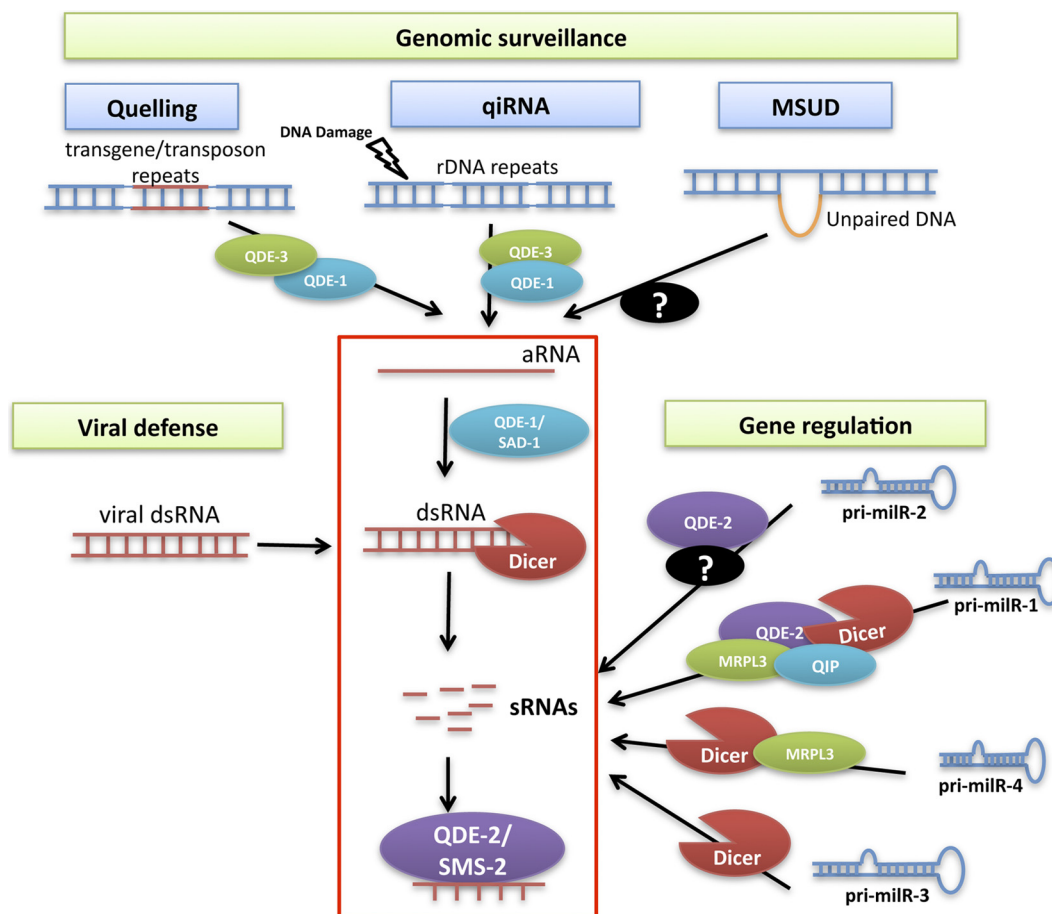


FIG. 1. sRNA biogenesis pathways and RNAi functions in *Neurospora*. See text for details.

ing that DCL-1/SMS-3 but not DCL-2 is specifically expressed during meiosis (1). SAD-2, a fungus-specific protein with no apparent domain, is required for meiotic silencing and can associate with SAD-1 *in vivo*. SAD-2 appears to function as a scaffold protein to recruit SAD-1 to the perinuclear region (5, 83). Similarly, DCL-1/SMS-3 and SMS-2 are localized in the perinuclear region, suggesting that this region is the major functional center for MSUD (1, 83).

Although meiotic silencing primarily utilizes a set of meiosis-specific RNAi genes, two recent studies showed that QIP, the exonuclease required in the quelling pathway, is also required for MSUD. These results suggest that quelling and meiotic silencing originated from the same ancestral pathways and are mechanistically linked (51, 92). It is now proposed that MSUD consists of two major steps: meiotic *trans*-sensing and RNAi-mediated silencing (46). The meiotic *trans*-sensing mechanism scans for the presence of unpaired DNA regions or significant sequence differences between the two homologous chromosomes during meiosis. Meiotic *trans*-sensing was initially discovered during studies of the *Asm-1* (*Ascospore maturation 1*) gene (4), which showed that the pairing of the *Asm-1* gene on homologous chromosomes is essential for its normal expression, indicating the existence of a *trans*-sensing mechanism that “senses” the presence/absence of paired DNA on homologous chromosomes. The failure to sense the presence of paired

DNA will trigger the production of sRNAs and silencing of the homologous DNA in the genome, a process that is mediated by SAD-1, SAD-2 SMS-2, and DCL-1/SMS-3 (52, 81, 82). Consistent with this model, DNA methylation in *N. crassa* appears to affect meiotic *trans*-sensing, but not meiotic silencing (72). The mechanism of meiotic *trans*-sensing is currently unknown, but genes known to be involved in stable long/short-distance pairing, recombination, and segregation steps of chromosomes during meiosis appear to be dispensable (46). Since the discovery of meiotic silencing in *N. crassa*, unpaired homologous DNA-triggered silencing in meiosis has also been observed in *Caenorhabditis elegans*, *Drosophila melanogaster*, and in mammals, but the silencing mechanisms in *Drosophila* and mammals appear to be independent from RNAi (46).

NOVEL TYPES OF sRNA AND PRODUCTION PATHWAYS IN *N. CRASSA*

DNA damage-induced qiRNA. Treatment of *N. crassa* cultures with various DNA-damaging agents induces the expression of *qde-2* mRNA and QDE-2 protein, suggesting the production of endogenous dsRNA after DNA damage. Analyses of the QDE-2-associated sRNAs revealed a novel class of sRNAs, named qiRNAs, because of their association with QDE-2. qiRNAs most originate from the rDNA locus, and

their production requires QDE-1, QDE-3, Dicer, and RPA, but they are different from quelling-induced siRNAs in three aspects: (i) qiRNAs originate from endogenous rDNA loci, (ii) they are about ~21 nt long, shorter than the typical 25-nt siRNA, and (iii) qiRNAs are induced in response to DNA damage (54). Despite these differences, recent studies suggest that the biogenesis of qiRNAs is similar to that of quelling-induced siRNA (53, 57) (Fig. 1). It is important to note that the rDNA locus is the only highly repetitive DNA region in the genome of wild-type *N. crassa* strain except when quelling is performed, which introduces another highly repetitive DNA locus. Therefore, the repetitive natures of rDNA and quelled loci are likely to be the common trigger for the production of aRNA and small RNA. The induction of qiRNA by DNA damage, therefore, suggests that replication stress on repetitive transgene loci results in siRNA production and quelling. Mutants deficient in qiRNA production exhibit increased sensitivity to DNA damage and an elevated protein synthesis rate upon DNA damage (54). These results suggest that qiRNA may contribute to the function of DNA damage checkpoints to arrest the cell cycle after DNA damage.

Study of *N. crassa* miRNAs reveals surprisingly diverse sRNA production pathways. miRNAs are important regulators of gene expression in animals and plants but were previously thought to be absent from fungi. By analyzing QDE-2-associated sRNAs using deep sequencing, at least 25 miRNAs were identified in *N. crassa* (55). Like conventional miRNAs, these miRNAs are predominantly derived from one strand of hairpin-like ssRNA precursors with strong preference for U at their 5' termini (55). In addition, miRNAs can repress the expression of endogenous genes, and the levels of the predicted miRNA target genes are upregulated in RNAi mutants. Furthermore, QDE-2 was found to be specifically associated with mRNAs that are predicted to be the targets of miRNAs. These results indicate that the *N. crassa* miRNAs are fungal miRNAs. However, the physiological importance of miRNAs remains to be determined.

Animal and plant miRNAs are produced by the well-defined canonical biogenesis pathways (3, 6). Genetic and molecular studies of *N. crassa* miRNAs, however, revealed surprisingly diverse biogenesis mechanisms (Fig. 1) and shed light on how eukaryotic sRNA can be made (55). These pathways combine different RNAi components, including Dicer, QDE-2, QIP, a putative RNase III domain-containing protein, MRPL3, and other unidentified nucleases, to produce different miRNAs. Among the four most abundant miRNAs, only *milR-3* maturation uses the canonical plant miRNA pathway, which only requires Dicers for pre-miRNA processing. Biogenesis of *milR-4* requires MRPL3 and is partially dependent on Dicer (55).

The analyses of the maturation of *milR-1* and *milR-2* miRNAs demonstrated that the Argonaute QDE-2 plays essential but distinct roles in two novel sRNA biogenesis pathways. For *milR-1*, the hairpin-containing primary *milR-1* is first processed by Dicer into double-stranded pre-*milR-1*. Subsequently, QDE-2 binds to pre-*milR-1* and recruits the exonuclease QIP to process the pre-*milR-1* into mature *milR-1*. In this pathway, QDE-2 functions as a scaffold but not a slicer in miRNA maturation. Although the production of most miRNAs is dependent on Dicer, *milR-2* is produced independently of Dicer.

Instead, the catalytic activity of QDE-2 is necessary for production of mature *milR-2*, suggesting that QDE-2, after binding to pre-*milR-2*, cleaves the miRNA* strand to allow the further processing of miRNAs by another unidentified nuclease (55). Interestingly, in both mice and zebrafish, the biogenesis of *miR-451* occurs through a similar pathway (17, 21), indicating that the novel sRNA pathways found in fungi also exist in higher eukaryotes.

Dicer-independent small interfering RNAs. Dicer-independent small interfering RNAs (disiRNAs) are another novel type of sRNA found in *Neurospora* (55). Like qiRNAs and siRNAs, disiRNAs are about 22 nt long and have a strong 5' U preference. disiRNAs are mapped to ~50 nonrepetitive DNA loci which contain genes or intergenic regions with no obvious sequence motifs. Importantly, the biogenesis of disiRNAs is independent of any known RNAi component, including Dicer proteins, indicating the existence of a completely unknown sRNA production pathway. Very little is known about the function of disiRNAs.

RNAi AND SMALL RNAs IN *MUCOR CIRCINELLOIDES*

M. circinelloides is a basal fungus of the clade zygomycete that has become an important fungal model system for RNAi and sRNA production. Transformation of *M. circinelloides* with self-replicative plasmids or with vectors for expression of inverted repeat transgenes results in activation of gene silencing pathways and production of sRNAs of two distinct sizes, 21 nt and 25 nt (26, 67–69). Of the two Dicer proteins, DCL-2 plays the major role in siRNA generation (26). *Mucor* has two RdRPs, and RdRP1 is required for transgene-induced gene silencing (68).

Deep sequencing of *M. circinelloides* sRNAs recently uncovered several classes of endogenous sRNAs (68). Interestingly, most of these sRNAs have sequences complementary to exons, and thus they are called exonic siRNAs (ex-siRNAs). ex-siRNAs are classified based on differential requirements for RdRPs and DCLs for their production. The biogenesis of most ex-siRNAs depends on RdRP1 and DCL-2, both of which are also required for transgene-induced silencing. These results suggest that RdRP1 may convert the exon-specific transcripts into dsRNAs, which are then processed by DCL-2. The second class of sRNAs requires DCL-2 and RdRP2 for their production, and the third class of ex-siRNAs requires both RdRPs and both Dicer proteins. The fourth class of ex-siRNAs requires both RdRPs but is mostly dependent on DCL-1 for biogenesis. In addition to ex-siRNAs, some DCL-dependent sRNAs originate from putative transposons and repetitive sequences. The differential roles of Dicer proteins and RdRPs in *M. circinelloides* sRNA biogenesis further indicate the diversity of fungal RNAi processing and sRNA production mechanisms.

SEX-INDUCED SILENCING IN *CRYPTOCOCCUS NEOFORMANS*

The human fungal pathogen *C. neoformans* also possesses a fully functional RNAi pathway, and transgene-expressed dsRNA has been successfully used to knock down gene expression in this organism (58). Recently, Heitman and colleagues discovered an RNAi-mediated posttranscriptional gene-silencing

ing mechanism that is specifically activated during sexual reproduction of the fungus, thus, the name, sex-induced silencing (SIS) (91). Like quelling, SIS requires the presence of a tandem array (>2 copies) of the transgene sequence, and the efficiency of SIS correlates with the copy number of the transgene. Although SIS can occur at low frequency during the vegetative growth stage of *C. neoformans*, its frequency is increased several hundredfold during the sexual phase. Importantly, SIS is abolished in *ago1* and *Rdp1* single mutants and in *dcl1 dcl2* double mutants, indicating SIS is an RNAi-related phenomenon.

Sequencing of the *C. neoformans* sRNA population revealed that in addition to the siRNAs derived from transgene loci, a significant portion of the siRNAs mapped to repetitive transposable elements and putative centromeric regions, which are rich in repetitive sequences and transposons (91). In the *Rdp1*, *Agol*, *Dcl1*, and *Dcl2* mutants, siRNA production from these loci was abolished. Importantly, the expression of the core RNAi components was highly induced during the sexual cycle, explaining why SIS is activated during the sexual cycle. Supporting the role for SIS in transposon control in *C. neoformans*, retrotransposons were found to be highly expressed during mating in *rdp1* mutants. Although how SIS recognizes repetitive sequences is not clear, its similarity to quelling and meiotic silencing in *Neurospora* suggests they may be similar pathways.

ROLE OF RNAi IN FUNGAL TRANSPOSON AND VIRAL DEFENSE

Transposon control in *N. crassa*. The silencing of repetitive transgenes by quelling suggests that quelling is a host defense against transposon invasion. Most of the examined *N. crassa* strains, however, lack functional transposons, due to the potent RIP process during the sexual cycle. The only exception is a LINE-1-like transposon, *Tad*, found in one African *Neurospora* isolate that was able to transpose in *Neurospora* (49). By introducing *Tad* into laboratory *N. crassa* strains, Nolan et al. showed that the repression of its transposition requires QDE-2 and Dicer but not QDE-1 or QDE-3 (70). These results suggest that *Tad* transposition may generate inverted repeats, from which dsRNA can be produced without QDE-1 and QDE-3.

Viral defense in *Cryphonectria parasitica* and *Aspergillus nidulans*. The role of fungal RNAi in viral defense was first established in the ascomycete filamentous fungus *C. parasitica*, the chestnut blight fungus. Nuss and his colleagues found that *Cryphonectria* hypovirus 1 (CHV1) alleviates the virulence of the fungal host by expression of a papain-like protease, p29. The presence of p29 suppresses hairpin RNA-induced silencing in *C. parasitica* (76). *C. parasitica* has two Dicer-like genes, *dcl-1* and *dcl-2*. The *dcl-2* mutant shows impaired growth after virus infection, indicating that DCL-2 is involved in virus suppression (77). *dcl-2* is essential for production of siRNAs derived from viral transcripts, and its expression is induced by virus infection (94). Although *C. parasitica* has four Argonaute-like genes, only *agl2* is required for the antiviral defense response, and its expression is also induced by viral infection (88). When the fungus was infected by a mutant hypovirus without p29, *agl2* and *dcl2* mRNAs accumulated to very high levels, suggesting that the virus suppresses RNAi by repressing

the expression of the core RNAi genes. In addition to their role in silencing viral replication posttranscriptionally, DCL-2 and AGL2 also promote viral RNA recombination in *C. parasitica*, resulting in the production of hypovirus defective interfering RNAs. Although the mechanism of the viral recombination is not clear, it is believed to be a viral control mechanism mediated by RNAi components (88, 93, 94).

The model organism *Aspergillus nidulans* also possesses a fully functional RNAi pathway, and the expression of inverted repeat-containing transgenes in this organism results in robust gene silencing (40). *A. nidulans* expresses one Dicer and one Argonaute protein, both of which are required for gene silencing. As in *N. crassa*, the RdRPs are not required for the inverted repeat triggered RNAi (39, 40). By infecting *A. nidulans* with mycoviruses, it was shown that viral infection suppresses the efficiency of RNAi, suggesting that an RNAi suppressor is encoded by this virus (39). Virus-derived siRNAs were maintained at a high level in the *Argonaute* mutants, confirming the antiviral role of RNAi.

APPLICATIONS OF RNAi IN FUNGI

RNAi provides an efficient and predictable gene silencing effect: recognition is based on the base pairing between siRNA and target RNA. Since RNAi-mediated silencing normally does not change the genomic structure of target genes, it serves as a good alternative to gene knockout and is extremely useful in studies of gene functions, especially when a gene is essential for cell viability (18). Moreover, RNAi-related methods may be used as tools in biotechnology to facilitate production of certain fungal metabolites by shutting down the expression of enzymes required for synthesis of end products (75). Although the RNAi pathway has been lost sporadically in various fungal species and lineages, most fungi possess this ancient mechanism (64). Three known methods can trigger RNAi-mediated gene silencing in fungi: expression of a hairpin RNA from a transgene, generation of dsRNA by convergent transcription from a transgene, and introduction of siRNA or dsRNA directly into the fungal cell (47, 57). Since the latter approach is not efficient for most fungi, the first two are the most commonly used.

Gene silencing via expression of a long hairpin RNA. The expression of a hairpin RNA specific for the gene of interest requires the creation of plasmid constructs in which transcription of an inverted repeat (up to 1 kb long) is under the control of a commonly used promoter (18, 35, 58). The expression of the inverted repeats in cells ensures the formation of a long hairpin RNA that is efficiently recognized and processed by Dicer. This approach has been widely used in many fungal species (45, 57, 58, 65). To facilitate the plasmid construction, several vectors have been developed, including a pSilent-1 vector that allows high-throughput RNA silencing (50, 80). The use of an inducible promoter to control the expression of the hairpin RNA offers significant advantages over constitutive promoters, especially when essential genes are the targets. The widely used quinic acid-inducible promoter in *N. crassa* and the alcohol dehydrogenase promoter in *A. nidulans* have been very effective in silencing gene expression in an inducer-dependent and dosage-dependent manner (7, 18, 35).

Dual promoters (convergent transcription). Although the inducible hairpin RNA silencing system is efficient, construction of plasmids containing the inverted repeats can be time-consuming, which limits its application to large-scale gene-silencing analyses. One alternative is to create plasmids with dual promoters. In this system, the gene of interest is flanked by two promoters that drive transcription in opposite directions. Thus, both the sense and antisense RNAs are transcribed simultaneously, and dsRNA should form (95). This method is suitable for high-throughput RNAi studies, in that the construct is relatively easy to make using systems like the Gateway RNAi vector system for fungi (66). The major disadvantage of this approach is that silencing is relatively inefficient compared to that with the inducible hairpin system, perhaps due to the low efficiency of formation of dsRNA (66, 73).

CONCLUSION

Studies of RNAi in fungi have made fundamental contributions to our understanding of RNAi and its functions. The recent discoveries of various small RNA biogenesis mechanisms in different fungi highlight the diversity of small RNAs and adaptation of RNAi pathways to different organisms and environments. Future RNAi studies of various fungal systems will no doubt shed light on the mechanisms, functions, and evolutionary origins of sRNA and RNAi pathways in eukaryotes.

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The space limitations of this minireview preclude a comprehensive description of RNAi-related work in fungi, and we apologize to those whose work we were unable to cite.

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